ORF0 could be reliably detected in ~50 copies in old world monkeys and thousands of copies in humans and great apes, but not in new world monkeys.

A critical question is whether ORF0 protein can be detected in non-engineered primate cells. Denli et al. (2015) provided evidence for the existence of the ORF0 protein using a combination of immunoprecipitation and mass spectrometry (MS). They overcame the issue of the mismatch between low ORF0 protein concentration and the limited dynamic range and sensitivity of MS by using polyclonal antibodies to enrich ORF0 protein. A second issue often encountered in MS analysis of short proteins is that, after digestion, there are often very few if any peptides amenable to MS sequencing, which need to be of a just-right length and well fragmented so that their sequences can be determined with high confidence. Denli et al. (2015) were able to obtain extensive fragmentation information almost entirely covering three tryptic peptides corresponding to ORF0 and its second exon (Figure 1). The MS detection was carried out on both overexpressed ORF0 protein and endogenous protein produced in human cells.

Just because a sequence is expressed does not make it a gene that encodes a functional protein. In this study, Denli et al. (2015) produced evidence suggesting a regulatory role for ORF0-encoded protein. Previous work had shown that an element driven by a promoter completely lacking LINE-1 sequences was active in retrotransposition, arguing strongly against a required role in *cis*. However, such a function might be provided in *trans*. Indeed, Denli et al. (2015) used a CAG-LINE-1 retrotransposition reporter element similar to those described earlier (Moran et al., 1996) to evaluate hopping frequency and showed that overexpression of ORF0 from a separate plasmid enhanced retrotransposition frequency by 41%. Thus, it seems likely that ORF0 plays some positive regulatory role in the retrotransposition process. It remains to be determined whether such a role of ORF0 is in any way related to its capacity in generating fusion protein containing host genomic sequences. Moreover, it would be interesting to see whether, and if so how, the ORF0 protein might functionally contribute to LINE-1 retrotransposition mechanistically.

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Evolutionary Reprograming of Protein-Protein Interaction Specificity

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Using mutation libraries and deep sequencing, Aakre et al. study the evolution of protein-protein interactions using a toxin-antitoxin model. The results indicate probable trajectories via "intermediate" proteins that are promiscuous, thus avoiding transitions via non-interactions. These results extend observations about other biological interactions and enzyme evolution, suggesting broadly general principles.

HEAD HEAL TEAL TELL TALL TAIL. This word game devised by Lewis Carroll requires moving from one word to another while keeping all intermediate words meaningful. It offers a nice analogy for a protein evolution model, where words represent functional proteins and mutations are word-to-word moves (Smith, 1970). It also represents one side of a debate, whether mutational navigation in sequence space from one protein function to another traverses via evolutionary intermediates that retain some functional features along the pathway to a new function. Because the evolution of new specificities in protein-protein interactions requires changes in at least two partners, the challenges for retaining functions that are vital for cell survival while evolving new ones may be more constrained (and more complicated) than in other

systems. How is cross-reaction between the evolving, homologous interaction partners evaded? What mutational trajectories do partners traverse while avoiding intermediate steps that may have negative biological consequences? In this issue of *Cell*, Aakre et al. (2015) utilize a model of toxin-antitoxin (TA) protein interactions that are essential for bacterial survival to study the problem systematically. Their results provide evidence for the preference for evolutionary paths involving biologically functional promiscuous intermediate steps, rather than switch-like trajectories that include non-interacting intermediates.

Bacteria typically include several chromosomally encoded paralogs of TA pairs in which an antitoxin neutralizes the toxin by interacting with it. Aakre et al. focus on the discrete problem of emergence of a new TA pair from an existing one. The specific ParD3/ParE3 interaction pair they chose for these experiments exhibits systemwide mutual exclusiveness, with almost no cross-reaction with other TA pairs that could complicate retrieval and interpretation of results.

Aided by a high-resolution crystal structure they solved for a specific ParD3/ParE3 complex, a 4-residue motif sufficient for reprogramming interaction specificity was identified. A mutation library was then constructed in which the one invariant Trp was retained while the three co-evolving positions of the motif were varied using only residues often found in natural ParD homologs. Fitness was approximated for intermediate stages in the path between one specific TA pair and another of different specificity using a competitive growth assay that allowed recovery of successful variants enriched over the time course of the experiments. For the ParD3 library, 252 variants were recovered that could effectively antagonize ParE3. As expected, repeating the competitive assay with another toxin, ParE2, produced a different set of 151 variants that neutralize this second toxin.

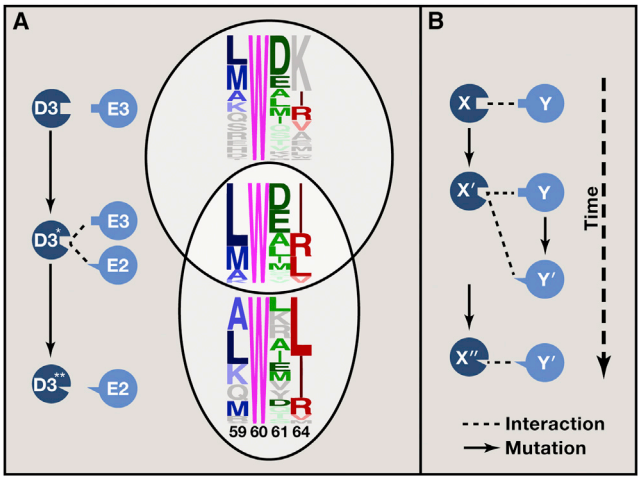

Figure 1. Reprogramming Specificity via Promiscuous Intermediates

(A) A Venn diagram showing the overlap between three sample sets of PraD3 antitoxin variants. Sequence logos represent the diversity in four specificity-determining positions that are overrepresented in ParD3 antitoxin variants that fit either ParE3 (the native toxin, upper), ParE2 (another toxin, lower) or both (middle). Amino-acid colors differ for each position; the darker the color, the more prevalent it is in the promiscuous motif. Grey residues represent cases in which the E3-specific logo or E2-specific logo includes residues that do not appear in the corresponding position in the promiscuous logo.

(B) The model suggested by Aakre et al. for reprograming protein-protein interaction specificity. An enabling, promiscuity-exerting mutation of protein X (X to X') allows protein Y to change its specificity determinant (Y to Y') and still bind both Xs. Protein X' then mutates (X' to X'') with an increase in specificity toward Y'. The protein-protein interactions are thus maintained throughout the evolutionary process. Features of this figure were adapted from Aakre et al. (2015).

The two antitoxin specificities are typified by distinct motifs in ParD3 specificity determinants (Figure 1A). While position 59 appears largely diffident in its variation pattern, position 61 is enriched for negatively charged residues for ParE3-specific variants, in contrast to small hydrophobic/positively charged residues in the ParE2-specific variants. Similarly, position 64 is enriched for positively charged residues in ParE3-specific variants, compared to small hydrophobic residues in ParE2 specific variants. Importantly, 31 variants exhibit dual-specificity toward ParE2/3, characterized by ParE3-like specificity at position 61 and ParE2-like specificity at position 64. Strikingly, evaluation of all alternative mutational trajectories between the two distinct specificities sampled shows statistically significant overrepresentation of traversal via promiscuous intermediates. Mutational trajectories also show significant enrichment for epistasis, rather than additive effect of mutations, consistent with similar findings in the evolution of enzyme-substrate interactions (Weinreich et al., 2006).

To investigate the important question involving co-evolution in interacting proteins, the authors performed another experiment traversing the sequence space from ParD3/ParE3 to ParD3*/ParE3*. Again, they found a prevalence in intermediate promiscuous variants, and, most importantly, that *all* presumed trajectories traversed via at least one promiscuous intermediate, suggesting the plausibility of this evolutionary path. Figure 1B summarizes these results, in which an X-Y interaction evolves to the orthologous X''-Y' interaction in at least three steps: (1) Mutation(s) in X to X' broadens specificity, allowing (2) Y to form a mutant, Y', that has the potential to interact with X as well as X', and finally (3) X' is mutated to X,'' narrowing its specificity to Y'.

Although reconstituting a natural history of protein repurposing is challenging and cannot be explicitly determined, this work contributes important initial observations toward this goal. Typically, mutation libraries sample a fraction of the sequence space. The approach used in this work allowed exclusion of infrequent, albeit viable trajectories, by focusing on the four most relevant positions and targeting only residues commonly appearing in contemporary proteins. Epistatic constraints and the occurrence of intermediates of modified or reduced function have been demonstrated for other types of models including in enzyme evolution, (Aharoni et al., 2005) and receptor-ligand evolution (Ortlund et al., 2007), and its practical implications have been exploited for protein-protein interaction engineering (Kortemme et al., 2004). Placed in this broader context, Aakre et al. provide new evidence for extending these conjectures to protein-protein interactions.

This work also suggests avenues for future research. For example, the

contributions of neutral drift and the impact of a few large-effect mutations versus many small-effect ones will need to be evaluated. Work on protein-protein interactions should be extended to other systems where cross-reaction is an issue, such as in other TA modules documented as cross-reacting (Zhu et al., 2010). Cross-reaction is also pertinent in other types of natural systems, as has been shown in some SH3 systems (Zarrinpar et al., 2003) and in the evolution of metabolic pathways (Kim and Copley, 2012). Ultimately, there are many other variables likely to be relevant in natural evolution that will surely be more difficult to ascertain in experimental systems. As with this work by Aakre et al., development of other new approaches may be essential for dissecting additional features in the evolution of protein-protein interactions.

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Bacterial Backstabbing: EF-Tu, Brute?

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Bacterial type VI secretion is an offensive and defensive weapon that utilizes a molecular warhead to inject toxins into neighboring cells. In this issue of *Cell*, Whitney et al. report a new class of toxin that disrupts the core metabolism of recipient cells and uncover a surprising requirement for EF-Tu.

Nowhere is life more fiercely competitive than in the invisible world of bacteria and other microbes. Vast in numbers, these diminutive creatures disable their competitors by assailing each other with a range of weapons that include dispersible small molecules (antibiotics) and protein toxins (e.g., colicins). Perhaps the most cunning weapon is the type VI secretion (T6S) system of *Vibrio*, *Pseudomonas*, and certain other Gram-negative bacteria, which forms a miniscule spring-loaded dagger—a phage-tail-like contractile apparatus, complete with a molecularly poisoned sharp tip—to instantaneously inject protein toxins at point-blank range into neighboring cells. Evoking the infamous Umbrella Murder, in which Bulgarian dissident Georgi Markov was assassinated by a ricin-laced projectile fired from an umbrella, the spear-gun-like T6S system fires into eukaryotic cells and bacteria alike, breaching their membranes and delivering toxic effector molecules with different modes of action. In this issue of *Cell*, Whitney et al. (2015) report that a recently discovered effector poisons cells differently from previously known effectors and, surprisingly, requires the translation elongation factor EF-Tu to intoxicate target cells.

The first functionally characterized T6S system, from *V. cholerae*, was revealed by its role in warding off predation by amoebae (Pukatzki et al., 2006), but T6S systems are increasingly viewed as part of an arsenal that bacteria use against one another. Indeed, bacteria can even be seen duking it out, repeatedly attacking and counterattacking in a process termed "dueling" (Basler et al., 2013). Characterized T6S effector molecules include lipases that target the bacterial membrane, peptidoglycan hydrolases that degrade the cell wall, and nucleases that act on the nucleoid (Figure 1A) (Durand et al., 2014). Structural and mechanistic studies of a recently discovered effector, called Tse6, by Whitney et al. (2015) reveal yet a different mechanism. Tse6 resembles diphtheria toxin and other toxins that transfer ADP-ribose from NAD^+ onto proteins to inactivate them, but Tse6 is a pure glycohydrolase that intoxicates cells by depleting them of cytoplasmic nicotinamide adenine dinucleotide (phosphate) ($NAD(P)^+$). Attacker cells expressing Tse6 are protected by its cognate immunity protein, Tsi6, which tightly plugs the Tse6 active site.

An enduring question is where in the target cell the warhead of effector proteins is initially delivered. Is it to the periplasm only, to the cytoplasm, or to both? In principle, the phage-tail-like tube of the T6S apparatus is long enough to penetrate 500 nm into a target cell (Basler et al., 2012; Ho et al., 2014), which could allow for direct delivery into the cytoplasm. But lipases and